



Increased Abundance Of *Gallionella* Spp., *Leptothrix* Spp. And Total Bacteria In Response To Enhanced Mn And Fe Concentrations In A Disturbed Southern Appalachian High Elevation Wetland

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Abstract

The Sorrento wetland hosts several Fe- and Mn-rich seeps that are reported to have appeared after the area was disturbed by recent attempts at development. Culture-independent and culture-based analyses were utilized to characterize the microbial community at the main site of the Fe and Mn seep. Several bacteria capable of oxidizing Mn(II) were isolated, including members related to the genera *Bacillus*, *Lysinibacillus*, *Pseudomonas*, and *Leptothrix*, but none of these were detected in clone libraries. Most probable number assays demonstrated that seep and wetland sites contained higher numbers of culturable Mn-oxidizing microorganisms than an upstream reference site. When compared with quantitative real time PCR (qPCR) assays of total bacteria, MPN analyses indicated that less than 0.01% of the total population (estimated around 109 cells/g) was culturable. Light microscopy and fluorescence in situ hybridization (FISH) images revealed an abundance of morphotypes similar to Fe- and Mn-oxidizing *Leptothrix* spp. and *Gallionella* spp. in seep and wetland sites. FISH allowed identification of *Leptothrix*-type sheath-forming organisms in seep samples but not in reference samples. *Gallionella* spp. and *Leptothrix* spp. cell numbers were estimated using qPCR with a novel primer set that we designed. Results indicated that numbers of *Gallionella*, *Leptothrix* or total bacteria were all significantly higher at the seep site relative to the reference site (where *Gallionella* was below detection). Interestingly, numbers of *Leptothrix* in the seep site were estimated at only 107 cells/g and were not statistically different in the late summer versus the late winter, despite dramatic changes in sheath abundance (as indicated by microscopy). qPCR also indicated that *Gallionella* spp. may represent up to 10% (3×10^8 cells/g) of the total bacteria in seep samples. These data corroborate clone library data from samples taken in October 2008, where 11 SSU rRNA sequences related to *Gallionella* spp. were detected out of 77 total sequences (roughly 10–15%), and where *Leptothrix* sequences were not detected. Analysis of this SSU rRNA clonal library revealed that a diverse microbial community was present at seep sites. At a 3% difference cutoff, 30 different operational taxonomic units were detected out of 77 sequences analyzed. Dominant sequence types clustered among the beta- and gamma- Proteobacteria near sequences related to the genera *Ideonella*, *Rhodoferrax*, *Methylobacter*, and *Gallionella*. Overall, results suggest that high metal concentrations at the seep sites have enriched for Fe- and Mn-oxidizing bacteria including organisms related to *Gallionella* and *Leptothrix* species, and that members of these genera coexist within a diverse microbial community.

INTRODUCTION

Wetlands are among the most vital of ecosystems, yet they continue to be threatened by development. In the southern Appalachian wetland system alone, it is estimated that only 300 hectares (ha) of wetlands remain out of the original 2000 ha that once existed. Of those 300 ha, most are considered to be disturbed, largely due to anthropogenic activity in the form of road construction, coal mining, urban and rural development, and agriculture (Semlitsch and Bodie 1998; Greco 2005). The southeastern United States, covering roughly 25% of the landmass in the continental United States, is home to nearly 50% of our nation's wetland ecosystems (Faulkner 2004); however, a paucity of information exists concerning the effect of

disturbance on microbial populations in these important and endangered southern wetland ecosystems.

One of the most important functions that wetlands provide is to promote nutrient and metal cycling, especially iron (Fe) and manganese (Mn) cycling (Walbridge 1993; Richardson 1995), and these reactions are often catalyzed by microorganisms (For example, see Weiss et al. 2003). In fact, the presence of Mn-oxidizing bacteria can speed up the rate of Mn oxidation up to five orders of magnitude over abiotic Mn oxidation (Tebo et al. 2005). *Gallionella* spp. and *Leptothrix* spp. are two metal-oxidizing bacteria commonly associated with metal cycling in aquatic and wetland environments.

Members of the genus *Gallionella* are known to oxidize Fe and have been detected in a range of Fe-rich environments, including acid-mine drainage waters in Carnoules, France (Bruneel et al. 2006), microbial mats at the bottom of a thermal vent in Crater lake, Oregon (Carlile and Dudeney 2000), salt and freshwater marshes in the Netherlands (Wang et al. 2009), and Fe-rich seeps along a California stream (Duckworth et al. 2008). *Gallionella* spp. are morphologically distinct with long branching twisted stalks coated with ferric oxides (Kucera and Wolfe 1957; Emerson and Weiss 2004; Kappler and Straub 2005) extending from a bean-shaped bacterium. Iron oxidation is believed to be associated with those stalks (Fortin and Langley 2005; Hallbeck and Pedersen 1995). *Leptothrix* spp. are another commonly found Fe- and Mn-oxidizing bacteria that have been detected in a variety of metal-rich aquatic environments, including lake bottom sediments (Stein et al. 2001), wells and groundwaters (Katsoyiannis and Zouboulis 2004), and wetlands (Emerson and Ghiorse 1992). *Leptothrix* spp. are strictly aerobic and chemoheterotrophic and can grow in the absence of Fe. The possibility exists that some species may grow mixotrophically, although, to date, no evidence exists conclusively demonstrating this idea (Spring 2006).

Leptothrix is unique in its ability to enclose itself in a Fe and Mn oxide encrusted sheath (Spring 2006). Members of this genus also tend to form thick woolly flocs and carpets that range in color from orange to dark brown depending on the extent of Fe and Mn oxidation occurring (Dexter Dyer 2003; Spring and Kampfer 2005; Spring 2006). It is also interesting to note that while there are four recognized species of *Leptothrix*, namely *L. ochracea*, *L. discophora*, *L. cholodnii*, and *L. mobiles*, *L. ochracea* is the most common *Leptothrix* spp. found in iron-rich freshwater environments (Emerson et al. 2010). Little is known about this species as it has yet to be successfully grown in laboratory cultures (Emerson et al. 2010).

The purpose of this study was to examine the microbial community structure around the Fe- and Mn-rich seep in the Sorrento wetland, a disturbed high elevation riparian wetland located near Boone, NC. Goals were to isolate important Mn(II) oxidizers, and to determine the relative change in abundance of specific Fe- and Mn-oxidizing bacteria (namely *Leptothrix* and *Gallionella*

species) in the seep site relative to the undisturbed reference site; thus, improving our understanding of these important bacteria that are facilitating remediation of excess Fe and Mn.

METHODS

Field Description

The Sorrento wetland (36°10'24.67"N, 81°37'55.01"W) is a small (roughly 18 acre) and isolated high elevation southern Appalachian riparian wetland that has been disturbed due to anthropogenic activity. It has been previously established that this disturbance has led to an increase in the levels of Fe and Mn in both the water and sediment in several areas of the stream and wetland (Greco 2005). Throughout the duration of this study, we observed several Fe and Mn seeps along the stream and within the wetland. These seeps were characterized by orange flocculent material (Figure 1) and oily sheens that shattered upon touch, often associated with Mn-oxidizing bacteria (Dexter Dyer 2003). Rocks within the streambed and downstream of the seeps were coated with a thick, orange biofilm layer, sometimes measuring up to two centimeters in thickness. Thick, woolly orange flocs were also present at various locations along the banks of the stream attached to weeds and debris within the water, and these sometimes extended up to several centimeters in length. None of the aforementioned features were present at the upstream reference site, along the undisturbed forested stream.

The Sorrento wetland is fed by a mountain tributary that passes through two impoundments and crosses under Sorrento Drive (in Blowing Rock, NC) before reaching the wetland. Site R is an upstream reference site located across Sorrento Drive and approximately 500 m upstream from the Fe and Mn seeps. Site S0 is located along the stream at the site of the largest Fe and Mn seep. Site W is located after the stream broadens and flows into the wetland itself.

Water Quality and Metal Analyses

Water samples were collected during October 2008 at sites R and S0 by submerging the mouth of sample bottles in the midpoint of the stream channel until the flasks were full. Water Quality Services Laboratory in Banner Elk, NC quantified the concentrations of Fe and Mn, and also provided trace metal grade sample bottles containing nitric acid to stabilize the dissolved metals immediately upon collection in the field. Temperature, pH, and dissolved oxygen (DO) were measured using a LaMotte #54183 water chemistry kit (Chestertown, MD). In September 2009 and March 2010, temperature and pH were measured using an Acorn® pH 6 portable pH meter (Oakton Instruments, Vernon Hills, IL). Biofilm material was digested in 5% oxalic acid and Fe and Mn concentrations were determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES) on a Varian 710 instrument.

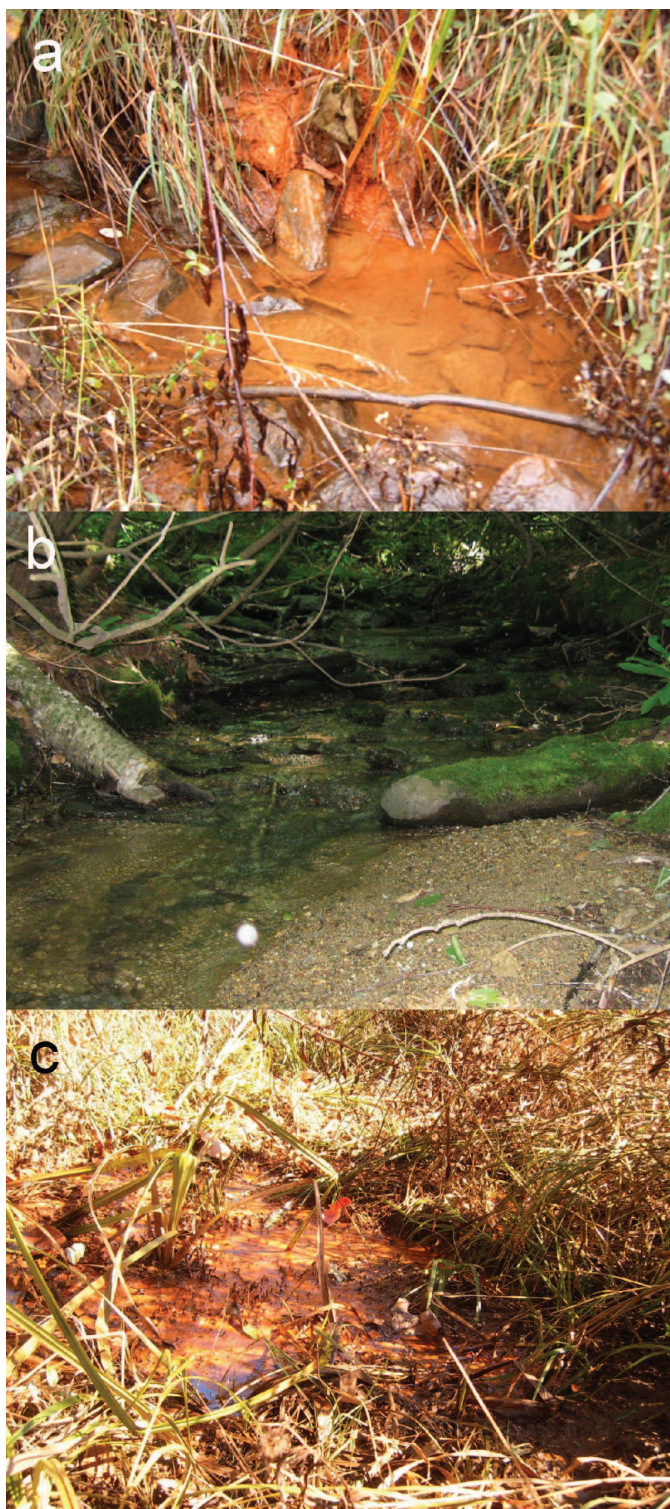


FIG. 1. Photographic images of sample sites. a) Rust colored floc associated with submerged grasses at the seep site (S0), leading to the Sorrento wetland. b) The relatively pristine reference site (R). Note the presence of a dense forest overstory and the thick undergrowth of rhododendron, as well as the lack of orange discoloration, submerged flocculent masses, or reed grasses. c) Rust colored submerged area of the wetland site (W), showing a thick growth of Fe- and Mn-oxidizing biofilm material, similar to that found at the S0 seep site (color figure available online).

Light Microscopy

Samples were collected from the soil-water interface at wetland, stream, and upstream reference sites by submerging a sterile Eppendorf™ tube into the water and using an ethanol sterilized spatula to guide the sample into the tube when necessary. Samples were transported immediately back to the lab. Slides were prepared by pipetting 40–50 μL of sample on to clean microscope slides. A coverslip was applied and edges were sealed with Maybelline® Express Finish Clear Nail Polish (New York). Slides were viewed using a Meiji MX light microscope (Santa Clara, CA) and images were taken using a SONY HD 6.1 Megapixel Handycam (New York, NY) with Martin Microscope Company MM9 Adapter (Easley, SC). Slides incubated *in situ*, as described below for FISH, were also prepared and visualized, but are not presented here.

Fluorescence Direct Counts (FDC)

Fluorescence direct counts of bacteria at the seep and reference sites were made using a protocol modified from previous studies (Adams and Ghiorse 1985; Emerson and Revsbech 1994). Briefly, samples were shaken in a solution of 0.1% (final concentration) sodium pyrophosphate ($\text{Na}_4\text{P}_2\text{O}_7 \cdot 10 \text{ H}_2\text{O}$, pH 7.0) for 10 minutes. Five microliters of the suspension were evenly spread over a 484 mm^2 area and stained with 1 $\mu\text{g}/\text{mL}$ (final concentration) DAPI (4,6-diamino-2-phenylindole). Citi-flour Antifadent Mounting Medium, AF1 (Electron Microscopy Sciences, Hatfield, PA) was added, and a coverslip was applied. Edges were sealed with Maybelline® Express Finish Nail Polish (New York). Samples were visualized using the 100x objective of an Olympus BX51 epifluorescence microscope. Dilutions were optimized to obtain an average of 10–30 cells per field of view and a minimum of 300 cells were counted per sample. Three different samples were collected from the seep and reference sites for direct counts.

Fluorescence in Situ Hybridization Microscopy

Microscope slides were anchored in a plastic tube rack and placed within the flow of water in the wetland, stream, and at the upstream reference site. After two weeks, slides were placed individually in sterile 50 mL conical tubes (Nunc™, Rochester, NY) and immediately transported back to the lab. Samples of water and sediment were also collected from the wetland, stream, and upstream reference sites as described above for light microscopy. Samples of oily surface sheen were collected from the wetland by submerging a sterile microscope slide beneath the water surface and lifting up to adhere the surface sheen onto the slide. Slides were placed in a sterile 50 mL conical tube (Nunc) and transported immediately back to lab. For homogenized samples, 50–100 μL of sampling material was applied to sterile microscope slides and left to dehydrate for several minutes. All slides were heat-fixed by passing over a flame for 1–2 seconds. The microscope slides were fixed using the protocol described by Osborn et al. (2005) for gram-negative bacteria. Briefly, slides were incubated in 50 mL conical tubes (Nunc™) containing ice-cold 4% paraformaldehyde solution for 6 hours

at 4°C. After 6 hours, slides were removed, briefly rinsed with 1 mL of ice-cold 1X phosphate-buffered saline (PBS) solution and washed 3 times with a large volume of 1X PBS. Fixed slides were resuspended in 50 mL of a 1:1 ratio of ice-cold 1X PBS and 96% ethanol. Fixed slides were either immediately hybridized or were stored at -20°C for up to 3 weeks before hybridization. Slides were hybridized using a protocol amended from previously described methods (Siering and Ghiorse 1996; Osborn et al. 2005). Briefly, fixed slides were placed at 46°C for 15 minutes and then incubated sequentially for 3 minutes each in 50% ethanol, 80% ethanol, and 96% ethanol. Slides were placed at 46°C for an additional 5 to 10 minutes until dry. One mL of freshly prepared hybridization buffer was prepared for each slide containing the following: 90 mM NaCl, 20 mM Tris-HCl, pH 9.5, 0.01% sodium dodecyl sulfate, and 15–30% formamide, depending on the probe used (Table 1). A mixture of 10 µL hybridization buffer and 1.5 µL of 50 µM fluorescently tagged oligonucleotide probe was added to each slide (see Table 1 for probe descriptions).

Slides were then placed in sterile 50 mL conical tubes (Nunc™) containing the remaining hybridization buffer and lined with lightweight tissue paper (VWR, West Chester, PA) and incubated horizontally for 8 hours at 46°C. After the incubation the slides were first rinsed in wash buffer (20 mM Tris-HCl, pH 9.5, and either 318 mM NaCl and no EDTA or 102 mM NaCl and 5 mM EDTA for slides hybridized with 15% or 30% formamide, respectively) and then incubated in wash buffer for 15 minutes at 48°C. Slides were then cooled by dipping in ice-cold sterile water for 3 seconds, air dried, and covered with aluminum foil to prevent exposure to light. Once dried, Fluoromount Antifadent (Sigma Aldrich) and glass coverslip were applied and slides were left to dry for an additional three hours. Slides were viewed within 24 h using a Zeiss LSM 510 Laser Scanning Microscope (Thornwood, NY), using an Helium Neon 633 nm laser and LSM 510 software.

SSU rRNA Clonal Library Construction

To determine the diversity and dominant members of the bacterial community at the site of the seep (S0) in the Sorrento wetland, a SSU rRNA clonal library was constructed. Samples of sediment were collected from the soil-water interface by submerging a sterile 50 mL conical tube (NUNC™) into the water and scooping up soil and water. All samples were capped and immediately transported to the lab for DNA extraction.

DNA extraction was done using UltraClean Soil DNA Kit (Mobio, Carlsbad, California) following manufacturer's protocol. This DNA (0.7 ng/µL, final concentration in the amplification reaction) was then used to amplify SSU rRNA genes using universal bacterial primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3'), and 1492r (5'-TACGHTACCTTGTACGACTT-3') (Lane 1991), and Taq polymerase (New England Biolabs, Ipswich, MA) according to the manufacturer's protocol. The cycling protocol included an

initial denaturation step of 94°C for 10 minutes, followed by 30 cycles of 94°C for 30 seconds, 53°C for 1 minute, and 72°C for 2 minutes, and an extension step of 68°C for 10 minutes. In attempt to reduce PCR bias 5 separate PCR reactions were combined.

The presence of the SSU rRNA gene was confirmed by gel electrophoresis. A roughly 1500 bp band corresponding to the expected size of the SSU rRNA gene were excised from all lanes, combined and purified using Promega Wizard SV Gel and PCR Clean-Up System following manufacturer's protocols. The purified product was then cloned into a TOPO 2.1 vector using Invitrogen TOPO TA Cloning® Kit (Carlsbad, CA), according to the manufacturer's protocol. Plasmids were isolated using the alkaline lysis method (Sambrook and Russell 2001). Plasmid DNA was diluted 1:50 and PCR amplified using Taq polymerase with M13 forward and reverse primers under the following cycling parameters: initial denaturation step of 94°C for 10 minutes, followed by 30 cycles of 94°C for 30 seconds, 53°C for 1 minute, and 72°C for 2 minutes, and an extension step of 68°C for 10 minutes. Eight µL of each reaction were run on a 0.5% agarose gel to verify product insert size. Ninety-five clones containing the correct insert size were grown up in LB broth containing 100 µg/mL ampicillin, frozen in 10% glycerol and sent to Beckman Coulter (Danvers, MA) for sequencing. Alignments of positive strand reads were created using the on-line SILVA aligner (Pruesse et al. 2007) and then exported from release 94 of the SILVA database in ARB (Ludwig et al. 2004). Chloroplast and chimera sequences were excluded from analysis. DOTUR was used to create rarefaction curves and to identify operational taxonomic units (OTUs) as defined by a 3% cutoff; i.e., 97% or greater identity (Schloss and Handelsman 2005). Eleven dominant OTUs (those with 2 or more sequence representatives) were identified (out of 77) and sequenced with primers M13 forward (-20), M13 reverse (-27), 357f (5'CCTACGGGAGGCAGCAG), 515r (5'TTACCGCGGCKGCTGRCAC), 926f (5'AACTYAAAKGAATWGRCGG), and 1098r (5'GGTYKCGCTCGTTGC). Sequences of dominant OTUs were assembled with Sequencher software (Gene Codes, Ann Arbor, MI), and were deposited into GenBank with accession numbers GU572364-74. Full length alignments were created as described above for positive strand reads. Phylogenetic trees were constructed using neighbor-joining and maximum likelihood methods in the PHYLIP software package (Felsenstein 2004). The number of sequences from the library that represented a particular OTU is given in parentheses.

Isolation of Mn(II)-Oxidizing Microorganisms

To isolate and identify Mn-oxidizing bacteria, samples of soil, water, and biofilm sediment were collected from R, S0, and W sites in sterile 1.5 mL Eppendorf™ tubes (Westbury, NY) using an ethanol-sterilized spatula when needed. Samples were immediately transported back to the lab for dilution and plating. Dilutions of 1:10, 1:100, 1:1000 and 1:5000 were

TABLE 1

List of probe sequences and labels used in FISH. Probes were constructed by MWG/Operon (Huntsville, AL). Percent formamide in the hybridization buffer is listed as % FA

Probe Name	Sequence	Label	Expected Target(s)	% FA	References
LD-1	5'-CTC TGC CGC ACT CCA GCT-3'	5'Cy5	<i>Leptothrix discophora</i> , <i>Sphaerotilus natans</i>	30	(Kampfer 1997; Loy et al. 2007; Siering and Ghiorse 1997)
EUB338	5'-GCT GCC TCC CGT AGG AGT-3'	5'FITC	Most Eubacteria	15	(Amann et al. 1990; Loy et al. 2007)
nonEUB	5'-ACT CCT ACG GGA GGC AGC-3'	5'FITC	NA, serves as negative control for FITC	15	(Amann et al. 1990; Loy et al. 2007)
nonEUB	5'-ACT CCT ACG GGA GGC AGC-3'	5'Cy5	NA, serves as negative control for Cy5	15	(Amann et al. 1990; Loy et al. 2007)

made in sterile PBS buffer plated on solid medium containing 15 g/L agar. A new medium, manganese oxidizer medium 1 (MnO1), was designed based on previous studies (Bräuer et al. 2010; Zinder et al. 1984), and contained the following (in mg L⁻¹): 12.0 NaCl, 1.2 KCl, 5.0 MgCl₂·6H₂O, 1.0 KH₂PO₄, 2.0 NH₄Cl, 1.0 CaCl₂·2H₂O, 0.155 CoCl₂·6H₂O, 0.155 ZnCl₂, 0.050 boric acid, 0.020 NiCl₂·6H₂O, 0.010 Na₂Mo₄·2H₂O, 0.1 mg MnSO₄·4H₂O, 3.0 MgSO₄·7H₂O, 0.1 CaCl₂·2H₂O, 0.01 CuSO₄·5H₂O, 0.18 AlK(SO₄)₂·12H₂O, 100.0 yeast extract, 1.5 ferric ammonium citrate, 500.0 casamino acids, as well as 100 µM MnCl₂, 0.2% v/v vitamin solution mix for J medium (Tebo et al. 2006) and 20 mM HEPES (pH 7.75). Several other types of media from the literature were used in this study including one herein called MnO#2 (Zakharova and Parfenova 2007), Fe/Mn (Rouf and Stokes 1964), and PTYP (Siering and Ghiorse 1996), as well as LB medium containing an additional 100 µM MnCl₂ (LB/Mn). Plates were incubated at 27°C in the dark until colonies appeared, usually within 2–12 days. Colonies were restreaked for isolation on the corresponding growth medium.

Isolated colonies were then tested for Mn oxidation by transferring a colony onto filter paper using a sterile toothpick and flushing with 20 µL 0.04% Leucoberbelin Blue (Sigma Aldrich, St. Louis, MO) (LBB), a redox indicator that is oxidized by Mn(III) to produce a bright blue color change (DePalma 1993). Bacteria that produced Mn oxides were grown up in liquid media corresponding to the solid media on which they were originally isolated. Cultures were incubated at room temperature in a shaking incubator until turbid (approximately 1 week). Gram stains were performed on each isolated colony using standard procedures. DNA was extracted using Qiagen DNeasy® Tissue Kit (Valencia, CA) following the protocol for gram-positive or gram-negative bacteria, depending on gram staining results. Resulting genomic DNA was used in a PCR reaction to amplify the SSU rRNA gene using universal bacterial primers 27f and 1492r (sequences are listed here; also see Lane 1991) using Crimson Taq Polymerase (New England Biolabs, Ipswich,

MA) following the manufacturer's protocols, and the following amplification reaction: an initial denaturation step of 95°C for 2 minutes, followed by 30 cycles of 95°C for 30 seconds, 53°C for 60 seconds, and 68°C for 90 seconds, followed by an extension step of 68°C for 5 minutes. Colonies that were filamentous in appearance under microscopic analysis and did not amplify with universal bacterial primers were considered fungal colonies. These isolates were not investigated further. PCR products were purified using Promega Wizard SV Gel and PCR Clean-Up System (Madison, WI) following manufacturer's protocol and sent to Cornell University Life Sciences Core Laboratories Center (Ithaca, NY) for sequencing with the same primers used for amplification. Only one sequence was related to sequences from the clone library (from isolate B8; 95% identity to either KWJ.F09 or KWJ.D01). The 27f-1492r PCR product from this isolate was cloned using an Invitrogen TOPO-TA cloning kit, sequenced with primers M13 forward (–20), M13 reverse (–27), 357f, 515r, 926f, and 1098r and assembled with Sequencher software as described above. Sequences from the isolates were deposited into GenBank with accession numbers GU572363 and HQ008928-31.

Most Probable Number Assays

To determine the abundance and quantity of culturable heterotrophic Mn-oxidizing microorganisms compared to the total culturable heterotrophic microorganisms present, MPN assays were performed. Soil, sediment, and water samples were collected from sites S0, R, and W in sterile 50 mL conical tubes (NUNC™) using a spatula sterilized in ethanol when needed. Samples were immediately transported back to the lab for dilution. Serial dilutions ranging from 10⁻¹ to 10⁻¹⁰ were made in sterile Corning® Costar® 96-well cell culture plates (Lowell, MA) with 8 replicates each. Next, 200 µL of liquid media and 20 µL of sample inoculum were added to each well in columns 1 through 10. Column 12 was inoculated with a Mn-oxidizing *Pseudomonas* sp. to serve as a positive control, and column 11 was left without sample inoculum to serve as a negative control.

We noticed during enrichment culturing that different types of media selected for completely different microorganisms. Therefore, two different types of media, MnO1 and LB/Mn, were used for MPN analyses, to attempt to culture a diversity of heterotrophic and Mn-oxidizing microbes with various metabolic needs. Plates were incubated at 27°C in the dark for 3–4 weeks. After incubation, 50 μ L of 0.04% LBB was added to each well in the top 4 rows to assess for Mn oxidation and 50 μ L 0.3% iodonitrotetrazolium chloride (INT) (Sigma Aldrich) (a redox indicator that is reduced in the presence of dehydrogenase to produce a bright red color) was added to each well in the bottom four rows to assess for heterotrophic metabolism. Plates were incubated for 24 hours and colorimetric results were applied to a downloadable MPN calculator (Curiale 2004) to determine the relative number of culturable heterotrophic microorganisms compared to those that oxidize manganese.

Real-Time Quantitative PCR Analyses

Samples were collected in quadruplicate in late summer/early fall (September 2009) and in late winter/early spring (March 2010) from the seep site and reference samples were collected for comparison. DNA was extracted using the FastDNA Spin Kit for Soil (MP Biomedical Sciences, Solon, OH), following the manufacturer's protocol, and was quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE). Primers specific for bacteria were identified from previous studies, and were examined for target diversity using the Probe Match tool on the RDP webpage (Cole et al. 2009), and for other traits using Primer Express[®] software (Applied Biosystems[™], Foster City, CA). The forward primer 338f 5'TCCTACGGGAGGCAGCAGT (Nadkarni et al. 2002) combined with reverse primer 518r 5'ATTACCGCGGCTGCTGG (Einen et al. 2008) were chosen as they gave broad coverage and a good amplicon size. For this study, two sets of primers were designed using ARB probe design (Ludwig et al. 2004), the SILVA release 94 database (Pruesse et al. 2007), Primer Express[®] software (Applied Biosystems[™], Foster City, CA), the Probe Match tool on the RDP webpage (Cole et al. 2009), and by taking into consideration optimal primer characteristics (Nadkarni et al. 2002). The first set was specific for *Leptothrix mobilis* and related species, including strain B8, isolated in this study, and clone KWJ_F09. *In silico* analyses indicated that the reverse primer, Lept 207r (5' TAGCGCGAGGCTCTTGC) had greater specificity than the forward primer Lept 28f (5'-TTGAACGCTGGCGGCAT). Together the primer pair matched 9 different *Leptothrix* sequences and only 12 non-target sequences out of roughly 1,000,000 total bacterial sequences in the RDP database. Both the forward primer Gall 55f (5' TG-CAAGTCGAACGGGCTTAG) and the reverse primer Gall 475r (5' TTCTCCGGTACCGTCATCCA) had good specificity. Together, they matched 10 uncultured *Gallionella* sequences (all from a study by Duckworth et al. 2008), 3 of the 11 sequences in the OTU represented by clone KWJ_C03, and did

not have any common non-target sequences. Both primer sets were challenged against non-target sequences (2 to 4 base pair mismatches) from clone libraries. At 64°C non-target clones were not amplified, whereas the target clones (containing sequences related to *Gallionella* spp. or *Leptothrix* spp.) were amplified. qPCR amplifications were performed on triplicate samples in Maxima[™] SYBR Green qPCR Master Mix (Fermentas, Glen Burnie, Maryland) containing 0.2 μ M of each primer and 0.1 ng/ μ L DNA.

Reactions were carried out using an Applied Biosystems[™] 7300 Real-Time PCR System (Foster City, CA) with the following amplification protocol: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C with 1 min at either 60°C for bacteria, or 64°C for *Gallionella* spp. or *Leptothrix* spp. After amplification, a dissociation protocol was added to verify dissociation profiles, and amplicons were run out on a 1% agarose gel containing GelRed (Phenix Research, Asheville, NC) to verify that only a single band was present. Plasmid DNA containing the target SSU rRNA gene from *Gallionella* spp. or *Leptothrix* spp. was used in the range of 10⁹–10² target copies per reaction to generate calibration curves for quantification as described previously (Fey et al. 2004). Copy number results were expressed on a basis of amount of DNA extracted per g of soil (wet weight). Our qPCR numbers may overestimate cell number up to 2- to 4-fold (Hou et al. 2010), since we used circular plasmid DNA for our standards. However, it's important to keep in mind that the overestimation caused by using circular plasmids may either be amplified or canceled by over-estimation or under-estimation due to other PCR biases (Suzuki and Giovannoni 1996; von Wintzingerode et al. 1997; Polz and Cavanaugh 1998; Smith and Osborn 2009; Hou et al. 2010;). Our own experiments have demonstrated a roughly 3-fold overestimation using circular plasmid standards compared to linearized standards. For example, in the summer, bacterial cell number was estimated around 2×10^9 using circular plasmids and 6×10^8 using linearized plasmids at the reference site, and 2×10^{10} using circular plasmids and 7×10^9 using linearized plasmids at the seep site. Thus, we have divided all copy number estimates for bacteria, *Leptothrix* and *Gallionella* by 3 as a rough estimate of the degree of overestimation (Hou et al. 2010), and to generate a more accurate estimate of genome copy number.

The number of copies in the sample, as determined by qPCR, was also divided by the average number of SSU rRNA gene copies per genome as a proxy for cell number (Fogel et al. 1999). The Ribosomal RNA Operon Copy Number Database (Klappenbach et al. 2001) was used to estimate the number of SSU rRNA genes copies for all bacteria (4.08), according to the database in April 2010. The NCBI Genome site was used to estimate SSU rRNA gene copy number for *Leptothrix* spp. as 2 copies, and for *Gallionella* spp. as 1 copy, based on the only sequenced genomes, *Leptothrix cholodnii* SP-6, accession number NC_010524, and *Gallionella capsiferriformans* ES-2, accession number NZ_ACSD00000000.

RESULTS

Water Quality and Metal Analyses

Measurements of dissolved oxygen at the seep site ranged from 7.4–9.0 mg/L, pH was between 6.25–7.9, conductivity was between 34–49 mS/cm, and alkalinity was between 18–20 mg/L CaCO₃. Water temperature was recorded as 7°C in October 2008, 20°C in September of 2009, and 6°C in March 2010. Also at the seep site, water samples contained roughly 100 μ M Fe and 6 μ M Mn, sediment samples contained 10 μ mol Fe and 7 μ mol Mn per g dry weight, and the biofilm material contained 250–700 μ mol Fe and 3–4 μ mol Mn per g wet weight. In contrast, the reference site contained only 1 μ mol Fe and 1 μ mol Mn per g dry weight in the sediment, and only 0.02–6.0 μ M Fe and 0.3–3.5 μ M Mn in the water samples.

Light Microscopy

Light microscopy of slides that were prepared using material from the S0 and W sites revealed two distinct morphotypes of bacteria with characteristics of *Gallionella* and *Leptothrix* species (Figure 3). Morphotype 1 consisted of long twisted helical *Gallionella*-like stalks, roughly 100–200 μ m in length (Figure 2a), some of which appeared to be encrusted with Fe and Mn oxides. Observations indicated that this morphotype seemed to dominate during winter months in both wetland and seep sites, although it was detected in all seasons. It also occurred in higher density in the wetland site compared with the seep site (data not shown). Morphotype 2 consisted of a dense tangle of long filamentous *L. ochracea*-like sheaths, ranging from 50 μ m to 300 μ m in length (Figure 2b), some of which appeared to be encrusted with Fe and Mn oxides. Observations indicated that morphotype 2 seemed to dominate in late spring to early fall months in both wetland and seep sites, although it was detected in all seasons. An example of morphotype 2 is also visible in the bottom right corner of Figure 2a. This morphotype appeared to be of higher density in the seep site compared with wetland sites and was also longer in length (roughly twice as long) in the seep site compared with wetland sites (data not shown). It is interesting to note that the majority of examples of morphotype 2 appear to be empty sheaths, devoid of cells. This is also indicative of *Leptothrix* spp., which can have as few as 7% of all sheaths containing live cells (Emerson and Revsbech 1994; Emerson and Weiss 2004). Neither morphotype 1 nor 2 were detected at the R site during the course of this study (Figure 2c).

In Situ Hybridization

FISH was employed to detect *Leptothrix* spp., and related organisms using the LD-1 probe, as described previously (Kamper 1997; Loy et al. 2007; Siering and Ghiorse 1997). Filamentous sheath-forming bacteria, each sheath measuring approximately 40–100 μ m in length, hybridized to the LD-1 probe in samples from the seep site (Figure 3a), but were not detected in samples from the reference site (Figure 3b). These cells are presumed to be *Leptothrix discophora*, or a related

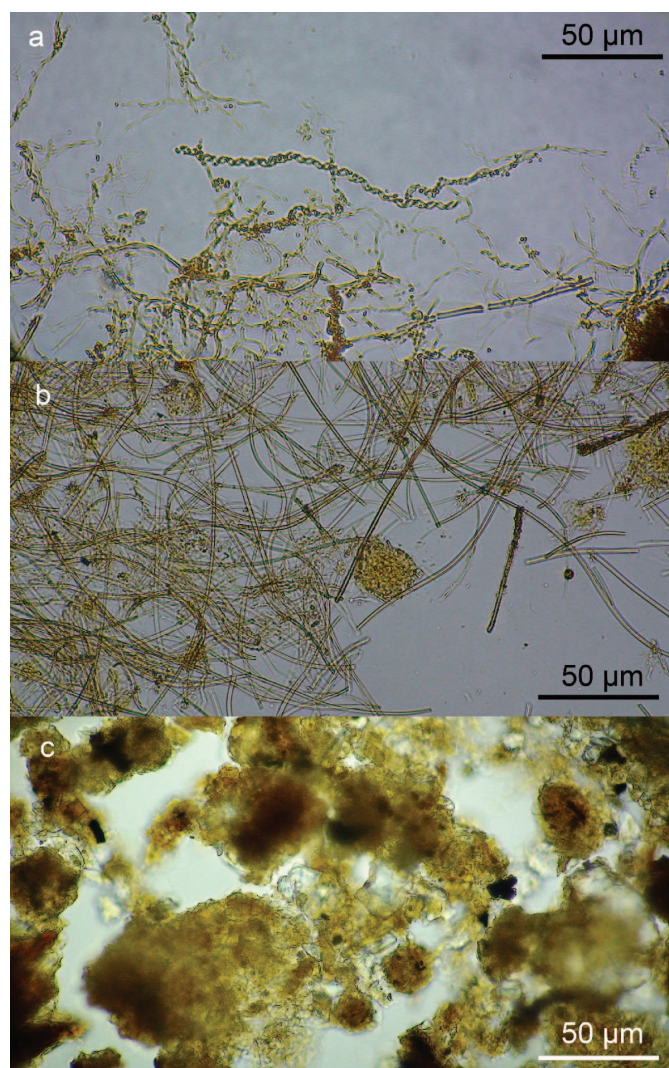


FIG. 2. Representative microscopic images of morphotypes detected in samples collected in winter (a), or summer (b), from the seep site, or from the reference site (c). a) Morphotype 1 from an S0 seep sample taken in February of 2009. Note the distinct helical structure, characteristic of *Gallionella ferruginea*. b) Morphotype 2 from an S0 seep sample taken in June of 2009. Note the distinct long filamentous sheaths, characteristic of *Leptothrix* spp. c) Soil aggregates visible in the R reference site from February 2009. Note the lack of morphotypes 1 or 2 (color figure available online).

organism such as *L. ochracea*. We noted that not all of the filaments fluoresced, indicating that a number of sheaths were devoid of bacteria, or contained low numbers of ribosomes.

FISH was also used to detect bacteria at both the seep and reference sites using the bacterial probe, EUB338. Small cocci were detected in the R site (Figure 3d). Coccoid, rod shaped, and long filamentous bacteria were detected in the S0 site, and large cocci were particularly abundant in these samples (Figure 3c). No fluorescence was detected in samples from either the R or S0 site using the nonEUB338 probe labeled with either FITC or Cy5 (data not shown).

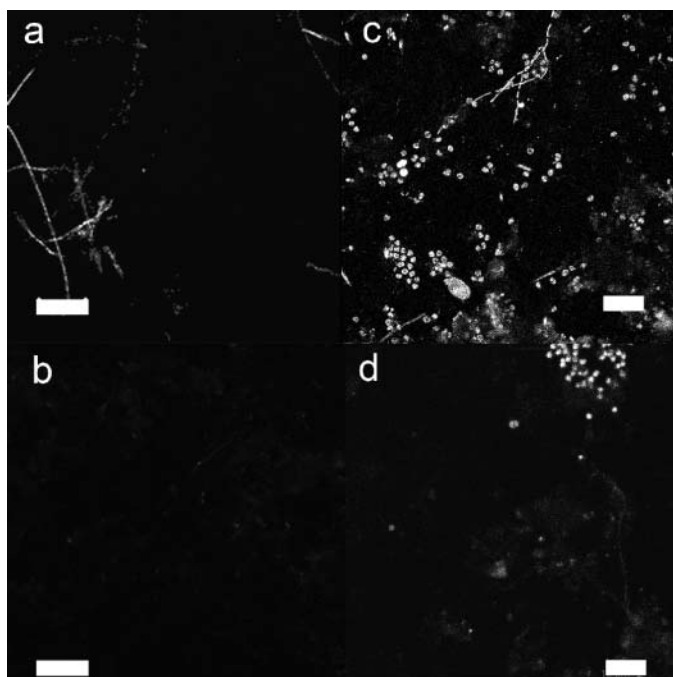


FIG. 3. FISH images from Sorrento Wetland. Fluorescence images were captured using samples from either the S0 site (a, c, top panels) or R site (b, d, bottom panels) that were hybridized with either the LD-1 probe labeled with Cy5 (a, b, left panels) or the EUB338 probe labeled with FITC (c, d, right panels). Scale bars represent 20 μm in all panels.

Isolation and Identification of Mn(II)-Oxidizing Microorganisms

A total of eight Mn-oxidizing bacteria, seven non-Mn-oxidizing bacteria, and five fungi capable of oxidizing Mn were isolated from floc material and water from the S0 seep site. SSU rRNA genes from five of the Mn(II)-oxidizing bacteria were successfully sequenced. Table 2 shows sequence identity information for the sequenced isolates, as well the growth medium and dilution factor used in plating. Colony and cell morphologies are also described. Blast analysis of these sequences revealed high identity (97–100%) with other sequences in the genera *Bacillus*, *Lysinibacillus*, *Pseudomonas*, and *Leptothrix* (Table 2). All isolates except for isolate B2, which is likely to be a *Bacillus* species, tested LBB positive as soon as colonies appeared on plates. Isolate B2 required 3–4 weeks before it tested LBB positive.

Quantification of Bacterial Abundance using Most Probable Number and Quantitative Real-Time PCR Analyses

To determine the relative abundance of culturable Mn-oxidizing heterotrophs compared to the abundance of total culturable heterotrophs in the Sorrento wetland and stream, most probable number (MPN) assays were performed. MPNs of total heterotrophs (cells per g wet weight) were determined to be

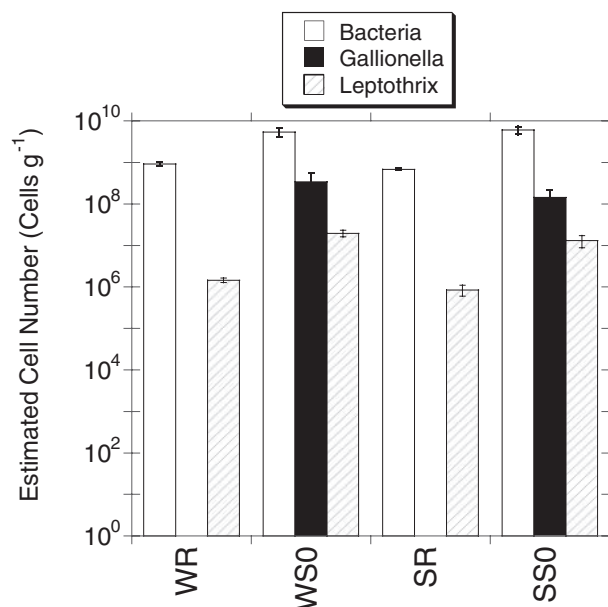


FIG. 4. Culture-independent quantification of bacterial abundance. Real-time qPCR quantification of SSU rRNA gene copy number of total bacteria and *Gallionella* and *Leptothrix* species in the Sorrento upstream reference site (R) and the Sorrento seep (S0) in late winter (W) or late summer (S). Data represent an average of quadruplicate samples and bars represent standard error.

2×10^5 for the reference site, and an average of 2×10^5 at sites downstream (S0 and W) when MnO1 medium was utilized. The most probable number of Mn oxidizers was significantly lower for the seep and wetland site (only 8×10^3 cells/g), and culturable Mn oxidizers were not detected in the reference site, or by using LB/Mn medium.

qPCR results of the copy number of total bacterial SSU rRNA genes in samples from the seep site collected in September 2009 and March 2010 respectively were estimated to represent 6×10^9 and 5×10^9 cells/g wet weight (Figure 4), and direct counts confirmed that cell number is in the 10^9 – 10^{10} range per gram wet weight (data not shown). For samples collected at the seep site in September 2009 and March 2010 respectively, quantification of SSU rRNA genes indicated the presence of 1×10^7 and 2×10^7 cells/g for a select clade of *Leptothrix* and related organisms, including the strain isolated in this study (B8), and 1×10^8 and 3×10^8 cells/g for a select clade of *Gallionella* including roughly 25% of clones clustering in the same OTU with clone KWJ_C03 (as described in the methods). In comparison, numbers of total bacteria at the reference site were lower, with cell numbers in samples collected in September 2009 and March 2010 respectively estimated at 7×10^8 and 9×10^8 cells/g wet weight. Estimates of *Leptothrix* in September 2009 and March 2010 yielded 8×10^5 and 1×10^6 cells/g wet weight, respectively, and *Gallionella* were not detected at the reference site.

TABLE 2
Identification and description of Mn(II)-oxidizing bacterial isolates

ID Name	Sequence Identity	Accession Number of Top Blast Hit	Percent Identity	Sequence Length	Dilution Growth Factor	Growth Medium	Colony and Cell Morphologies	Gram ±
B1	<i>Lysinibacillus</i> sp.	GU827539.1	99–100%	590	1:100	Solid Fe/Mn	Dark tan round colonies, 5 mm, rod shaped	+
B2	<i>Bacillus</i> sp.	GU458276.1	97–100%	391	1:100	Solid MnO1	White filamentous colonies, 4–5 mm, Mn oxidation is delayed 3–4 weeks, rapidly spreading, rod shaped	+
B5	<i>Pseudomonas fluorescens</i>	GU358073.1	100%	560	1:1000	Solid MnO1	Brown round colonies with darker center, 1 mm, rod shaped	–
B7	<i>Bacillus</i> sp.	GQ128226.1	99–100%	440	1:1000	Solid MnO1	Brown colonies, <1 mm, thick Mn oxide crust, rod shaped	+
B8	<i>Leptothrix mobilis</i>	NR_026333.1	99%	1349	1:1000	Solid MnO1	Brown colonies, 1–2 mm, lack distinct edge, rod shaped	–

Analysis of Bacterial Community through SSU rRNA Clonal Library Construction and Phylogenetic and Diversity Analyses

A SSU rRNA clonal library was constructed and analyzed in order to determine the bacterial diversity at the Fe and Mn seep and identify dominant bacterial community members. DOTUR analyses revealed 30 distinct operational taxonomic units (OTUs) at a 3% difference cutoff out of 77 total sequences (Figure 5). 19 singleton OTUs (those having only one sequence representative) were identified. Most notable were those sequences affiliated with groups containing known Mn and/or Fe oxidizers including *Sphaerotilus* (1), *Burkholderiales* (1), and *Sphingomonas* (1). Dominant OTUs (as defined as those containing two or more sequence representatives at a 3% difference cutoff) included sequences in the *Betaproteobacteria*, *Gammaproteobacteria*, *Acidobacteria* and *Cytophagia* classes of bacteria (Figure 6). Sequences in the *Betaproteobacteria* clustered near sequences from cultured strains of the genera *Ideonella*, *Rhodospirillum*, *Methylobacter*, *Gallionella*, and one clustered with uncultured sequences from other Fe-oxidizing cultures or rhizosphere biofilms (accession numbers for unpublished studies, see Figure 6). Sequences in the *Gammaproteobacteria* clustered near *Methylobacter* and *Methylosarcina*. One dominant OTU also clustered phylogenetically with *Acidobacterium*-related clones from soil, including clone BacB.003 (Hansel et al. 2008) and one clone that was associated with an iron and manganese nodule (He et al. 2008). Finally, the last dominant OTU clustered in the *Bacteroidetes* group near *Terrimonas ferruginea* with clones retrieved

from aquatic environments (see Figure 6), including clones from the north basin (Glockner et al. 2000), from ground water (Longnecker et al. 2009), and from a humic lake (Newton et al. 2006).

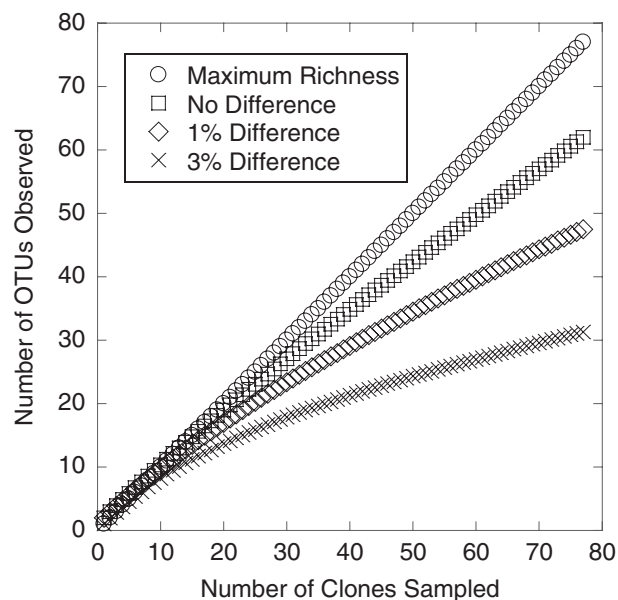


FIG. 5. Rarefaction curves from DOTUR analysis of the clone library of the Sorrento seep site showing the number of operational taxonomic units estimated from the 77 clones sequenced. OTUs are shown at a 3%, 1%, and 0% difference cutoff. In addition, the theoretical maximum richness is shown for comparison.

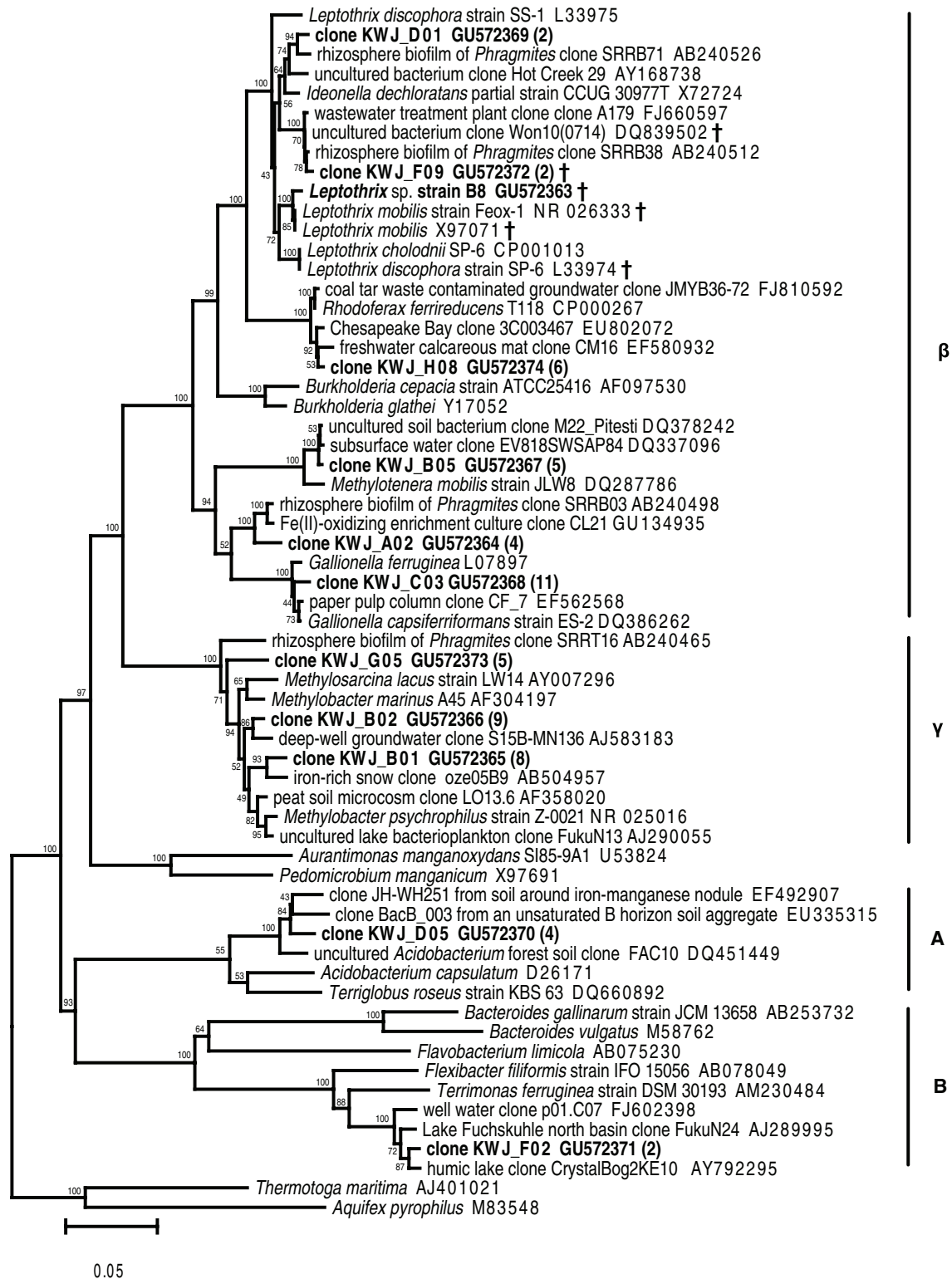


FIG. 6. Neighbor-joining tree inferring the phylogenetic relationship between the cultured Mn(II)-oxidizing *Leptothrix* sp. strain B8 (bolded and italicized) and the dominant OTUs found in a clone library (bolded) of the Sorrento seep site. The number of sequences from the library that represented a particular OTU is given in parentheses. Sequences in this tree that match the *Leptothrix* primer set are denoted by a dagger (†). Top blast hits to the isolate and clones were included, as well as a broad range of described species. Alignments were created using the on-line SILVA aligner and then exported from ARB. The dendrogram was created using PHYLIP. Bootstrapping values are shown for all nodes. The analyses were supported with maximum likelihood analysis (data not shown). *Thermotoga maritima* and *Aquifex pyrophilus* were used as the outgroup.

DISCUSSION

Water Quality and Metal Analyses

Fe and Mn concentrations for the S0 site exceeded limits set by the North Carolina Department of Water Quality (NCDWQ). Results support previous studies (Greco 2005; Pitchford 2007) reporting extremely high levels of Fe and Mn, that exceed both the EPA published limits known to sustain aquatic life (Emerson et al. 1999) as well as the NCDWQ limits. All other indicators were within the normal range for the NCDWQ standards. Due to high Fe and Mn concentrations, the health of the Sorrento wetland for macroinvertebrates and other aquatic life has clearly been compromised, as previously established (Greco 2005; Pitchford 2007).

Compared to a study of another freshwater Fe seep (Emerson et al. 1999), the concentration of Fe in the soil at Sorrento was much lower (10 versus 900 $\mu\text{moles/g}$), whereas the concentration of Fe in the biofilm material (250–700 versus 300–6,300 $\mu\text{moles/g}$) was only somewhat lower than that measured by Emerson et al. Concentrations of Fe in the water were well within the typical range reported in other metal-rich systems. For example, Kasama et al. measured 256 μM Fe in water samples using ICP (Kasama and Murakami 2001), and Emerson et al. (1999) measured 150 μM or 250 μM (Emerson and Revsbech 1994) in water samples using the ferrozine method. Thus, the Fe seep at the Sorrento wetland is comparable to other Fe-rich circumneutral freshwater habitats.

Microscopy

Observations from light microscopy demonstrated the dominance of two different morphotypes, thought to be Fe- and Mn-oxidizing *Leptothrix* spp., most likely *L. ochracea* and *Gallionella* spp., in the S0 and W sites. These morphotypes were not observed in the R site. FISH analysis also showed *Leptothrix*-type organisms present in the S0 and W sites, but not in the R site. However, FISH images from the S0 and W sites showed far fewer numbers of *Leptothrix*-type bacteria when compared with light microscopy results, perhaps due to low numbers of cells (e.g., empty sheaths as noted by Emerson and Revsbech 1994; Emerson and Weiss 2004), or the presence of inhibitors, such as humic acids (Tebbe and Vahjen 1993). Preliminary spectroscopy results indicated high concentrations of humic substances absorbing at 230 nm (results not shown) in all sample sites tested, which could also lead to undetectable FISH signals.

Also noteworthy, were the seasonal fluctuations in abundant morphotypes. Empirical observations from light microscopy indicated that *Leptothrix* spp. dominated in late spring and summer months, while *Gallionella* spp. dominated in cooler late fall and winter months. Fe and Mn concentrations are known to fluctuate seasonally, due especially to seasonal hydrological variations. These variations could alter the magnitude and persistence of redox gradients, affecting metals such as Fe and Mn (Thompson et al. 2006). Because *Leptothrix* spp. and *Gallionella* spp. both require specific oxygen and redox gradients (Blothe and Roden

2009; Emerson and Revsbech 1994), these seasonal variations could affect them also, causing seasonal blooms and declines in populations. It is also possible that sheath formation by *Leptothrix* spp. may be stimulated in summer, leading to the visual appearance of dominance, while cell number remains the same.

FISH images using the universal eubacterial EUB338 probe labeled with FITC showed an abundance of several morphotypes, including large cocci, in the S0 and W sample sites that appeared to dominate the slide image, especially in the S0 site. None of the bacterial isolates were cocci, nor are species within the genera *Leptothrix* or *Gallionella*, so it is likely that the large cocci observed represent a bacterium (or bacteria) not yet identified by this study.

An analysis using the probe match tool of RDP (Cole et al. 2009) revealed that the LD-1 probe is an exact match to a wide variety of sequences from organisms in the family *Burkholderiaceae*, including organisms closely related to *Leptothrix*, *Aquicola*, *Ideonella*, and *Rubrivivax*. Among the clones from the Sorrento wetland, the probe is not an exact match to any of the sequences, but has one, two, three, or four mismatches to clones KWJ_D01, KWJ_F09 and KWJ_H08, and the isolate *Leptothrix* sp. strain B8 respectively. In addition, LD-1 has three mismatches with the *Sphaerotilus*-affiliated sequence in the library. Probe LD-1 has been shown to readily hybridize with members of the genus *Sphaerotilus*, including *Sphaerotilus natans* (Siering and Ghiorse 1997). Morphologically, *S. natans* is also a long, sheath-forming bacterium, but it has a distinct sausage-link shape to it and tends to be found in organic-rich wastewater. Therefore, it is possible, though unlikely, that the long filamentous morphotypes picked up by probe LD-1 belong to the genus *Sphaerotilus*. Additionally, the possibility that other organisms outside of the genus *Leptothrix* were hybridized cannot be ruled out. In fact, at least one other similar study (Haaiker et al. 2008) has also hypothesized that species related to, but outside of, the genera *Leptothrix* and *Gallionella* may be responsible for iron oxidation in some freshwater seeps. Interestingly, in a recent study of another similar freshwater seep, Bruun and colleagues also observed *Leptothrix*-like sheaths, but did not detect any sequences related to *Leptothrix* in clone libraries, although sequences clustering near both *Leptothrix* and *Ideonella* sequences were retrieved (Bruun et al. 2010).

Currently, very little is known about organisms in the genus *Ideonella*, but they appear to have diverse metabolic capabilities, being able to aerobically fix nitrogen and anaerobically reduce chlorate (Noar and Buckley 2009). Neither of the two described species of *Ideonella*, *I. azotifigens* and *I. dechloratans*, is known to form a sheath. However, of the described species of *Sphaerotilus* and *Leptothrix*, only one has maintained the ability to form sheaths in culture (Spring 2006), thus it is possible that other organisms, such as *Ideonella* may have the ability to form sheaths *in situ*. This issue could be resolved by designing and testing species-specific FISH probes to target *Ideonella*, *Sphaerotilus* and/or other sequences found in the clone library,

or *Leptothrix*-type sequences, if these could be retrieved from more extensive libraries of the site.

Isolated Strains

Members of the genera *Bacillus* and *Pseudomonas* are known Mn oxidizers and are readily cultured from environmental samples. Although members of the genus *Lysinibacillus* have not previously been reported to oxidize Mn, this genus was until recently grouped with other *Bacillus* species (Ahmed et al. 2007); thus, it is not known if any of the previously reported Mn oxidizers may cluster with *Lysinibacillus*. The delay in Mn oxidation for isolate B2 (related to *Bacillus* species) is likely due to the time required for spore production, since *Bacillus* spores have been shown to directly oxidize Mn (II) to Mn (IV) (Bargar et al. 2000; Francis and Tebo 2002). Interestingly, strain B1 (related to *Lysinibacillus* species) was capable of oxidizing Mn readily on agar plates, suggesting that Mn oxidation may occur prior to spore formation in this strain. Members of the genus *Leptothrix* are Fe and Mn oxidizers, often found in wetlands and environments rich in Fe and Mn. And, while a *Leptothrix*-related strain was cultured, sequences with high identity (>95%) to the isolate were not retrieved from molecular analyses, including those from a previous study (Pitchford 2007). This suggests a low abundance of the cultured representative in Sorrento wetland, and reveals limitations in traditional culturing methods.

Quantification with MPN and qPCR

Compared to other studies, fewer bacteria, and fewer metal oxidizers were culturable at the Sorrento site, with only 10^3 – 10^5 cells/g cultured out of the 10^9 cells/g that are estimated to be present. For example, Blothe and Roden cultured up to 10^9 aerobic heterotrophs/mL and up to 5×10^5 Fe oxidizers/mL using MPN analyses at a similar seep site (Blothe and Roden 2009). In another study of a Mn-rich estuary, up to 10^4 aerobic heterotrophs and 10^4 Mn oxidizers were cultured out of roughly 10^6 estimated using qPCR (Bräuer et al. 2010). It is not known whether the low numbers obtained here may be due to differences in site characteristics, media type, or microbial population.

Estimates of cell number using direct counts or quantitative real-time PCR are within the range expected for Fe- and Mn-oxidizing biofilms, or other types of biofilms. Theoretically, it has been estimated that a dense biofilm could contain up to 10^{11} cells/mL (Edwards et al. 2000), and numbers up to 10^{11} cells/mL have indeed been measured in cultured biofilms (Prigent-Combaret et al. 1999). One study examined dense microbial mats in sulfidic systems and found numbers on the order of 10^{10} cells/mL (Engel et al. 2004). However, for the most part, numbers for other similar Fe- and Mn-oxidizing biofilms in circumneutral pH wetland areas ranged in the 10^8 – 10^9 cells/mL (Emerson and Revsbech 1994), which is in line with our estimates of 10^9 cells/g.

Numbers of *Leptothrix*, *Gallionella*, and total bacteria were all significantly enhanced in the metal-rich seeps compared to

the reference site. Not surprisingly, *Gallionella* spp. were not detected at the reference site, confirming microscopic observations. This may indicate that the concentrations of Fe at the reference site, which were roughly two orders of magnitude lower than those of the seep site, were too low to support growth by *Gallionella*. In contrast to *Gallionella*, *Leptothrix* spp. were detected at the reference site. This confirms a previous study which found one sequence affiliated with *Leptothrix* (97% identity to *L. discophora*) in a small ($n = 20$) clone library of the same reference site (Pitchford 2007). Interestingly, sheaths were not detected in microscopic images of the reference site, perhaps indicating that environmental conditions (possibly the dramatic increase in Fe) are inducing sheath formation. There were no statistically significant differences between summer and winter samples, although a more rigorous examination over a longer time frame may reveal distinct seasonal differences. The small increase in estimated *Gallionella* abundance at the seep site in winter versus summer moderately supports empirical observations from microscopic images. For *Leptothrix*, however, estimated numbers in winter were two fold higher than in summer, when they would be expected to be much lower based on microscopic observation. Again, this may suggest that environmental conditions are influencing sheath formation and that sheath formation (but not cell number) may be greater in the summer months.

On the other hand, primer design may also play a role. Due to low sequence coverage (only 77 sequences), we did not have access to a large database of sequences of indigenous *Leptothrix* strains; thus, the primer pair may not target the organism responsible for the abundant sheath formation. Overall, qPCR data corroborated microscopy results and indicates that the influx of reduced metals can support dense and thriving bacterial biofilm communities that harbor an abundance of Fe- and Mn-oxidizing bacteria.

Analysis of the SSU rRNA Clonal Library

Analysis of the SSU rRNA clonal library generated from the S0 floc material showed a diverse community, with 30 different OTUs identified at a 3% difference cutoff. The most dominant OTU, with 11 sequence representatives, clustered near *Gallionella ferruginea*, a known Fe and Mn oxidizer. The next most dominant OTUs with 9, and 8 sequence representatives, clustered near *Methylobacter*, a known methane oxidizer. This may be an indication of the redox conditions in the sample, as it was collected near the oxic/suboxic soil horizon, where Fe and Mn oxidizers are known to thrive (Emerson and Weiss 2004). Among the singletons, one affiliated with the Mn(II)-oxidizing genus *Sphingomonas* (Francis et al. 2001) and one affiliated with the Fe(II) oxidizing genus *Sphaerotilus*. Interestingly, none of the other clone sequences could be associated with known Mn or Fe oxidizers, although some could be associated with clones from similar environments. Perhaps this is an indication that there is a low diversity and/or low number of Mn oxidizers at the site, consistent with culturing and MPN results. On the other

hand, the phylogenetic diversity of Fe and Mn oxidizers continues to expand (Santelli et al. 2010; Templeton et al. 2005; Weiss et al. 2007). Given the low percentage of total culturable bacteria, it is likely that some of the clone sequences may have come from organisms that are capable of Mn and/or Fe oxidation.

According to the DOTUR analysis of the sequence data at a 3% difference cutoff, the percentage of unique OTUs was estimated at roughly 63% (30 unique OTUs out of 77 total clones). This suggests lower diversity than in a preliminary study of a subset of the same clones analyzed in this study, where RFLP analysis identified 62 different phylotypes from 68 of the clones (Johnson 2009). In either case, analyses indicate that the community diversity found at the seep site may be potentially higher than other environments that are considered to harbor very diverse communities including cave environments, where 9 unique restriction patterns were identified by RFLP (Northup et al. 2003) or where 173 unique OTUs (at a 1% difference cutoff) were identified out of 414 total sequences (Porter et al. 2009), metal-rich lake sediment where 18 out of 78, 6 out of 44, and 22 out of 84 unique restriction patterns were identified by RFLP (Stein et al. 2001, 2002), and in sediment samples from gulf waters in Papua New Guinea where 33 unique sequences were identified out of 91 total sequences (Todorov et al. 2000). Rarefaction curves in Figure 5 have not yet begun to level out after 77 sequences, indicating that we have not covered the full diversity of sequences found at this site.

CONCLUSION

Results from this study have shown a very diverse community of microorganisms coexisting in the Sorrento wetland. Culture results demonstrated the presence of several bacteria and fungi that are capable of oxidizing Mn(II). MPN data indicated an increase in Mn-oxidizing heterotrophic bacteria in the Sorrento wetland compared with upstream reference sites. Microscopic observations as well as culture and molecular data all indicated that numbers of Fe- and Mn-oxidizing bacteria, including *Leptothrix* and *Gallionella* were enhanced in the seep site relative to the reference site. Based on these data we can conclude that the increase in Fe and Mn concentrations in the wetland have not adversely affected diversity, but have enriched for Fe- and Mn-oxidizing bacteria. It is probable that these Fe- and Mn-oxidizing bacteria are at least partially responsible for the deposits of Fe and Mn oxides that are present throughout the Sorrento wetland. More work is needed to determine for certain if the abundance of *Gallionella* and/or *Leptothrix* might change seasonally or temporally, or if there are environmental conditions, such as pH contributing to the observed changes in sheath abundance.

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